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## INTRODUCTION

We have proposed the hypothesis that *carcinogen-induced changes in the microenvironment constitute a third class of carcinogenic action distinct from those leading to mutation or proliferative advantage*. Carcinogen-induced microenvironments are postulated to increase the number or susceptibility of epithelial cells to transformation, exert a selective force on initiated cells and/or are conducive to progression. If the microenvironment induced by carcinogens can shape the features and frequency of neoplastic phenotypes, then the carcinogen 'fingerprint' may be envisioned as being built by first laying a foundation of genotypic alterations that expand in the context of a microenvironment that is the result of carcinogen-induced phenotypic change. Understanding this aspect of carcinogenesis is important since certain microenvironment alterations might be amenable to modulation, which in turn could provide the means to modify cancer progression. The proposed studies are intended to obtain further evidence for this hypothesis.

We have studied the effects of a known breast carcinogen, ionizing radiation, on the microenvironment of the mouse mammary gland. We have showed that rapid and global remodeling of the mouse mammary gland extracellular matrix occurs and that it is mediated by the activation of the multipotent cytokine, transforming growth factor- $\beta$ 1 (TGF- $\beta$ ), a potent regulator of both epithelial and stromal function. Activation can be detected at doses as low as 0.1 Gy. The extracellular matrix of the irradiated mammary gland undergoes rapid remodeling that includes the novel expression of tenascin and collagen type III. Blocking TGF- $\beta$  with neutralizing antibodies inhibits radiation-induced collagen type III expression, providing functional confirmation of TGF- $\beta$  activity. Based on these studies, we concluded that exposure to a carcinogen such as radiation can elicit persistent changes in gene expression by non-initiated cells.

By creating chimeric mammary glands consisting of normal or irradiated mammary epithelium in normal or irradiated stroma, we found that the irradiated stroma impedes epithelial maturation. The first aim of the present grant is to test whether radiation-induced TGF- $\beta$  activity regulates this phenotype. The effect of the irradiated stroma may relate to the well-documented age dependence of radiogenic breast cancer. For example, if radiation-induced microenvironment delays the development of the gland in differentiating past a critical check point, then the size or sensitivity of the carcinogen-susceptible population may be increased. Alternatively, radiation-induced TGF- $\beta$  may be a selective force that allows expansion of initiated cells resistant to TGF- $\beta$ . To test whether preneoplastic cells progress more readily in an abnormal stroma, we propose in aim two to create chimeric glands consisting of preneoplastic epithelium in normal versus irradiated stroma.

We predicted that, given the known age dependence of radiogenic mammary cancer in both mice and women, the character of the microenvironment would change as a function of both radiation exposure and mammary development. Our third objective is to compare the radiation-induced microenvironment of adult and immature mice, with particular attention to the expression and activity of TGF- $\beta$ . The regulation of TGF- $\beta$  activation and activity in vivo is not well-understood. We have begun this study by examining the effect of development, hormonal status, and differentiation on TGF- $\beta$  activation.

**CURRENT STUDIES**

**Aim 1: Determine the role of TGF- $\beta$  in the inhibition of mammary gland development by irradiated stroma by using neutralizing antibodies to knockout TGF- $\beta$  activity during outgrowth.**

**The Effect of the Irradiated Stroma on Mammary Gland Outgrowth**

Preliminary studies demonstrated that radiation exposure induced both active TGF- $\beta$  immunoreactivity and collagen III expression in cleared fat pads with kinetics similar to those found in intact mammary gland. TGF- $\beta$  peaks at 1 day and persists for 7 days, whereas collagen III peaks at 3 days and is completely resolved by 7 days. Based on these changes in microenvironment, 12-week old gland-free host animals were irradiated whole body to 4 Gy with  $^{60}\text{Co}$ -gamma radiation 1, 3 and 7 days prior to transplantation. Both fat pads of 7 adult animals were transplanted with mammary gland fragments (1 mm<sup>2</sup>) from control mice or mice irradiated with 1.5 Gy. The animals were killed after 52 days. The fat pads were evaluated as wholemounts and classified regarding the degree of filled fat pad, the percent of fat pads with end buds and the percent with side branching independently by my collaborator Dr. Sandra Haslam (Michigan State University, E. Lansing).

**TABLE I: Effect of the Irradiated Stroma on Mammary Outgrowth**

DAYS POST FP IRRADIATION	% FP FILLED		% FP W/ ENDBUDS		% FP W/ SIDE BRANCH	
	CONTROL	1.5 GY	CONTROL	1.5 GY	CONTROL	1.5 GY
None	86 (9)*	60 (12)	35	24	44	54
1	98 (8)	80 (11)	68	72	16	8
3	76 (9)	48 (8)	22	74	22	2
7	80 (11)	78 (13)	36	69	18	14

*\* Number of fat pad (FP) 'takes' (14 possible) analyzed in each experimental group.*

These data included in the proposal are summarized in Table I. Although irradiated donor tissue was somewhat inhibited in filling the 3 day irradiated host, both donors grew comparably in other irradiated hosts. This suggests that the growth or number of donor cells were not significantly decreased by radiation. In addition, the irradiated hosts were able to support full growth of the mammary gland, which suggests that there was no persistent effect on adult hormonal function. As mentioned above, radiation-induced remodeling occurs in locally irradiated mammary glands (Ehrhart and Barcellos-Hoff, unpublished) and therefore the changes in microenvironments are mediated by paracrine and autocrine routes, rather by changes in endocrine function.

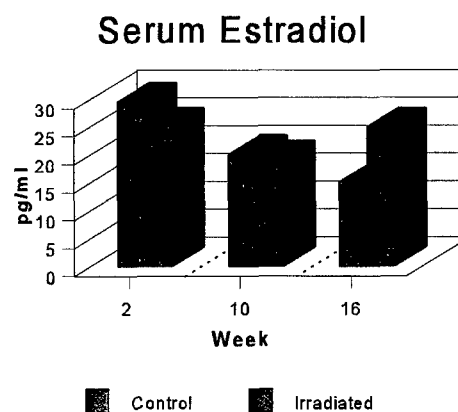
Surprisingly the irradiated donor tissue in the unirradiated hosts did not give rise to significantly hyperplastic or dysplastic growth. This data contrasts with the studies by Ethier and Ullrich on which

we based the experimental design (Ethier and Ullrich, 1982). However, since their study transplanted to 3 week old newly cleared fat pads versus our experiment with 12 week old hosts, two factors may have altered the expression of preneoplastic lesions: the "wound" environment of the newly cleared fat pad versus our healed site, and the influence of pubertal hormones versus the adult estrous cycle on the growth/selection of mammary outgrowths.

Nonetheless the irradiated stroma has a profound effect on the character of mammary outgrowth from both control and irradiated donor tissue. Most of the groups transplanted into irradiated hosts had significantly more endbuds and less lateral branching. Both endpoints are indices of the maturity of the mammary epithelium and indicate that the outgrowth, though capable of proliferation, retains the immature phenotype. Outgrowth was most compromised when both host and donor tissue are irradiated, regardless of the time at which the host was irradiated. These effects are again not due to donor irradiation since irradiated tissue transplanted to a normal host exhibit development comparable to control tissue. Indeed, unirradiated donor tissue exhibited greatly increased endbuds and decreased side branching when implanted into hosts irradiated one day before. This effect was diminished with increasing time between radiation and transplantation since the frequency of fatpads with endbuds in the 3 and 7 day irradiated hosts transplanted with normal donor tissue were comparable to the unirradiated host, although they still exhibited reduced side branching.

Irradiated epithelium transplanted to unirradiated hosts is capable of mature differentiation and lateral branching. Tissue implanted 1 day post irradiation, when TGF- $\beta$  activity is the greatest, shows the most immature differentiation. The phenotype of either irradiated or normal epithelium transplanted to irradiated stroma lacks lateral branching and contains endbuds, even though the fat pad is fully filled. Interestingly a transgenic mouse harboring a mammary epithelial specific promoter driving constitutively active TGF- $\beta$  overexpression has recently been reported to have a hypoplastic morphology very similar to the irradiated transplants (Pierce et al., 1995). Expression of a constitutively active protein was necessary since the wild-type construct, even when driven by the strong MMTV promoter, did not exhibit a phenotype. Moses and colleagues postulated that the major effect was due to inhibition of proliferation but also suggested that there may be subpopulation variation in susceptibility to TGF- $\beta$  regulation.

We have repeated and extended the experiment described in the preliminary data. The wholemounts from this experiment are currently being analyzed. The immature phenotype exhibited in the first experiment was proposed to be susceptible to transformation in a manner similar to that of the pubertal mammary gland. This raised the issue of whether host whole body irradiation altered ovarian functions. We therefore examined the ovarian competence of irradiated hosts by measuring plasma levels of estradiol. Serum was pooled from 3-4 animals and measured at the Clinical Endocrinology Laboratory, School of Veterinary Medicine, University of California, Davis. Levels of estradiol from irradiated animals do not differ significantly from the mean of the control at 2, 10 and 16 weeks post irradiation.

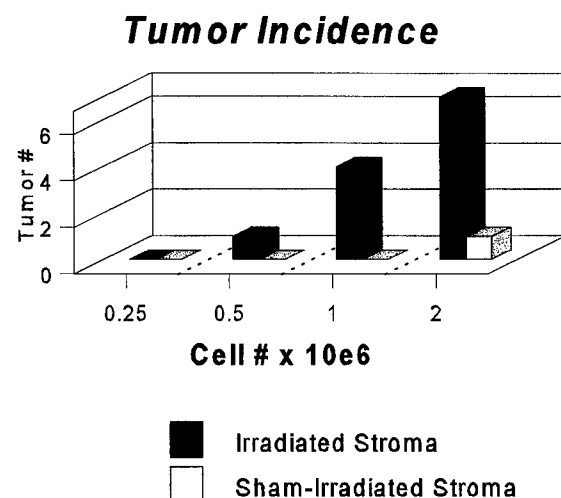


**Aim 2: Determine the effect of sham versus irradiated fat pads on the carcinogenic potential of epithelia treated with chemical carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA).**

The objective of this aim was to evaluate the role of the irradiated stroma on neoplastic progression, i.e. whether the abnormal stroma "pushes" premalignant cells towards malignancy. We had considered the use of preneoplastic cell lines in designing the original experiments but lacked preliminary data. This option is attractive from the view of animal conservation since donor animals are not required. Recent studies indicated that the COMMA-D mammary epithelial cell line might be a feasible alternative. COMMA-D cells have a mixed morphology, are capable of normal outgrowth formation in cleared fat pads, retain the potential to produce differentiated products and exhibit low tumorigenicity (Danielson et al., 1984). However a number variants have been selected both in vivo and in vitro that are highly tumorigenic (Medina et al., 1987). Recent studies have shown that COMMA-D population is clonal based on characteristic p53 mutations in both alleles (Jerry et al., 1994). We asked whether the neoplastic potential COMMA-D cells could be promoted by transplantation to irradiated stroma.

**Effect of the Irradiated Stroma on Progression**

To test the effect of the irradiated stroma on progression, different numbers of COMMA-D mammary epithelial cells were injected into 6 cleared fat pads of normal or irradiated adult mice. Irradiated hosts received 4 Gy whole body 24 hr prior to injection. Wholemounts were prepared at 6 weeks post-injection. Ductal-type outgrowths were formed at low frequency when transplanted to either control or irradiated tissue. Tumors formed in 1/6 fatpads in sham-irradiated tissue transplanted with 2 million cells while 7 tumors were found in 6 irradiated fat pads. Tumor formation depended on the number of cells injected. We postulate that the irradiated stroma promotes the tumorigenic capacity of the COMMA-D cells by facilitating progression. We will repeat this experiment this year, confirm tumor types by histology, and prepare tissue for immunohistochemistry in order to determine the character of the microenvironment.



**Aim 3: Define the radiation-induced microenvironment as a function of mammary gland developmental status.**

We have demonstrated that remodeling of the irradiated mouse mammary gland microenvironment is mediated in part by TGF- $\beta$  (Ehrhart et al., 1997). TGF- $\beta$  is an important regulator of differentiation, proliferation and extracellular matrix composition. It has been postulated to play both a positive and negative role in cancer development and progression, which suggests that determining its physiological regulation and activity in particular tumors may provide interesting targets for therapy (Reiss and Barcellos-Hoff, 1997).

TGF- $\beta$  is secreted as a latent complex that is unable to bind to TGF- $\beta$  receptors until the biologically active 24-kD mature TGF- $\beta$  is released; this activation is considered to be the critical regulatory event for TGF- $\beta$  function. Radiation exposure elicits rapid and persistent activation of TGF- $\beta$  *in vivo*. We postulated that aberrant TGF- $\beta$  activation by ionizing radiation affects mammary gland development and neoplastic progression by perturbing the balance between the stroma and epithelium.

The effects of TGF- $\beta$  in the mammary gland are complex and not well understood. In order to better understand the role of TGF- $\beta$  during mammary gland growth and development prior to examining the effect of developmental status on the response to radiation, we examined the distribution and abundance of active TGF- $\beta$  in Balb/c mice during normal mammary development. Using an immunostaining protocol that preserves endogenous latent TGF- $\beta$  and antibodies that discriminate between latent and active TGF- $\beta$ , we determined that in normal adult mammary gland latent TGF- $\beta$  is abundant but active TGF- $\beta$  is restricted to epithelial structures. During puberty endbuds contained cells that were strongly positive for active TGF- $\beta$  while subtending ducts were not. In adult nulliparous animals, low levels of active TGF- $\beta$  were evident throughout the epithelium during diestrous, while during estrous, both the abundance and localization of active TGF- $\beta$  changed dramatically. In particular, some luminal epithelial cells were strongly stained while adjacent cells were negative. These cells may represent a distinct subpopulation. During pregnancy, both active and latent forms of the protein decreased gradually through to lactation. These data suggest that activation of latent TGF- $\beta$  is highly regulated and potentially controlled by hormone action. It is paradoxical in light of TGF- $\beta$ 's inhibition of proliferation that the highest levels of TGF- $\beta$  appear concomitant with periods of active growth, i.e. endbuds and estrous.

Interestingly, we have found that the expression of latent and active TGF- $\beta$  in cultured COMMA-D cells is also heterogeneous. The primary culture contains both polygonal and bipolar cells that segregate both spatially and in regards to trypsin sensitivity. Many of the cells stain for latent TGF- $\beta$  but active TGF- $\beta$  is found primarily in ridges of bipolar cells. When the two morphologically distinct cell types are isolated by differential trypsinization, only the bipolar cells are immunoreactive and the majority are not stained for active TGF- $\beta$ . The polygonal cells are negative for either the latent or active form of TGF- $\beta$ . Thus it seems that the latter cells require the presence of bipolar cells to produce TGF- $\beta$  and the bipolar cells require the presence of the polygonal cells to activate TGF- $\beta$ . We have injected these cell types into normal and irradiated fat pads to determine whether their tumorigenic potential is different from the mixed parent population.

## CONCLUSIONS

Our first year of funding has supported experiments leading to two important and novel observations. We proposed that the effects of carcinogens on the phenotype of host tissue could contribute to the progression of initiated epithelial cells. Such a role for the abnormal stroma created by radiation exposure has been confirmed using the COMMA-D mammary cell line. This study supports our hypothesis that the microenvironment elicited by carcinogen exposure can act as a promoter of neoplasia and therefore, the possibility that microenvironments may be a future target for therapeutic intervention or cancer prevention.

TGF- $\beta$  is a potential mediator of breast cancer progression and an important target for therapy. In order to understand the impact of TGF- $\beta$  action on mammary carcinogenesis it is critical to

understand its physiological mechanisms of activation and the consequences of its activity during development. Our initial studies of the physiological regulation of TGF- $\beta$  activation in normal murine mammary gland have shown that activation is highly restricted and is differentially regulated by differentiation and estrous. The postulated role of TGF- $\beta$  as a key regulator of normal mammary proliferation will be examined in using specific hormonal manipulations. Its' role in progression will be evaluated using in vivo neutralization of TGF- $\beta$  action in radiation chimeric mammary glands. These studies will provide the critical background for determining the consequences of TGF- $\beta$  activation in the irradiated mammary gland and its role in neoplasia.

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